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Defects in repair of double strand chromosome breaks (DSBs) are emerging as important factors both in familial and sporadic breast tumors. Just as a bacterial model for single-base mismatch repair lead to rapid identification of human homologues with roles in carcinogeneis, development of a bacterial model for DNA DSB repair will provide important insights into the role this system plays in breast tumor development. This project has focused on developing a bacterial model for DSB repair by characterizing the enzymatic apparatus needed to initiate DNA replication on recombination intermediates. We describe how the initiation of PriA helicase activity on the DNA fork is influenced by both fork structure and by single-strand DNA binding protein. The two factors may direct PriA helicase to translocate along the lagging-strand template of forked DNA to facilitate PriA's 3' to 5' helicase activity to unwind the DNA duplex and promote loading of the replicative helicase DnaB, a central step in assembly of the replisome. These data indicate that PriA is an initiator protein that responds to DNA damage and assembles the components necessary for repair by replication, maintaining the integrity of the chromosome and preventing potentially catastrophic loss of information and mutation.

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Introduction:

Genetic defects in breast tumors frequently involve mutations in both oncogenes and tumor suppressor genes. Genes involved in the repair of DNA can be classified as tumor suppressor genes, but thus far only genes required for one type of DNA repair, single-base mismatch repair, have been fully characterized in humans. While defects in these genes appear to play a role in a small number of breast tumors, defects in repair of double strand chromosome breaks (DSBs) are emerging as important factors both in familial and sporadic breast tumors. We have focussed on development of a bacterial model for repair of DSBs by replication coupled to homologous recombination, and such a system will likely provide insight into the mechanism of DSB repair in humans. The reconstituted system for bacteriophage Mu replication by transposition has been an invaluable tool in this process. During Mu transposition, strand exchange catalyzed by the phage-encoded transposase MuA leads to formation of a branched DNA structure with a potential replication fork at either end of the transposing DNA element, similar to the branched intermediates created during homologous recombination. Bacterial proteins including the replicative helicase DnaB and DNA polymerase III holoenzyme then assemble a replisome at one end this substrate and commence semi-discontinuous DNA synthesis from one end to the other. Like replication coupled to recombination on the bacterial chromosome, initiation of bacteriophage Mu replication is independent of the chromosomal initiator protein DnaA, suggesting that bacteriophage Mu may harness the cellular apparatus required for coupling replication with recombination. Our finding that the *Escherichia coli* PriA protein was required for Mu replication by transposition both *in vivo* and *in vitro* supported this hypothesis. Previous to our work, PriA had been hypothesized to couple replication with homologous recombination based on genetic evidence and on the role of PriA in assembly of a primosome for bacteriophage fX174 complementary strand synthesis. Our work provided the first definitive biochemical evidence that PriA could couple replication with recombination.

Report Body

Summary of Research Progress:

As a result of Jessica Jones' graduation and departure from Dr. Hiroshi Nakai's laboratory, I have been selected to carry on the project of identifying host proteins required for bacteriophage Mu replicative transposition.

Mu replicative transposition occurs through a series nucleoprotein structures initially created through the recombination events of strand transfer catalyzed by MuA transposase. The strand transfer complex (STC) is remodeled through the actions of host protein ClpX, a molecular chaperone [1]. The modified nucleoprotein complex (STC2) is necessary for the formation of a new nucleoprotein complex (prereplisome) catalyzed by additional, yet unidentified, host factor(s), referred to as Mu replicating factor (MRFα2). Completed work of Dr. Jones indicates that the prereplisome directs the binding and helicase activity of PriA on the Mu fork [2, 3]. PriA intiates the formation of a primosome to promote Mu DNA synthesis resulting in the formation of a replicated product, the cointegrate. It was previously demonstrated in the Nakai lab that the crude *E. coli* extract, MRFα2, responsible for disassembly of STC2 and formation of the prereplisome could be separated into two separate fractions, referred to as MRFα2A and MRFα2B [4].

I began this project year setting up a replication system of purified proteins as an assay system for the remaining host protein components (MRFα2A) needed to catalyze replicative transposition in bacteriophage Mu. I acquired the skills to perform the *in vitro* Mu transposition system, first established by Kyoshi Mizuuchi (Mizuuchi reference), to successfully measure Mu replication through the formation of cointegrate product . This assay system is critical for the purification of MRFα2A.

Upon establishment of this assay system, I began preparation of the partially purified MRFα2A. This protocol requires crude lysate fromWM433/ClpX- *E. coli*. A single ammonium sulfate precipitation is performed resulting in a partially purified extract referred to as MRF (Mu replicating factor). MRF is passed through a heparin agarose column, which results in two essential components, MRFα and MRFβ. MRFβ consists of the primosome assembly proteins required for Mu replication while MRFα consists of at least three factors, molecular chaperone ClpX and host factor(s) referred to as MRFα2. Resolution of MRFα2 on a Q-sepharose column resulted in two separate fractions that were called MRFα2A and MRFα2B. But the partially purified extract from the WM433/ClpX- strain contained excessive nucleases that often degraded the strand transfer substrate (STC). In the absence of MRFα2B cointegrate formation could not be detected but it was unclear whether this was due to inability of MRFα2A to support cointegration formation without MRFα2B or because of insufficient STC substrate due to nucleases. Findings indicated that MRFα2A was comprised of a heat labile protein factor and MRFα2B was a heat stable tRNA. MRFα2B was implicated as a possible nuclease inhibitor,

helping to maintain a stable level of STC substate for replication. Levels of STC degradation made assessment of MRFα2B difficult. Crude extract preparation of MRFα2A was switched from WM433/ClpX- strains to a DH5α strain which is endA1 and therefore lacks the potent endonuclease I. This was to minimize the effects of nuclease observed in partially purfied extracts used in the *in vitro* system. I prepared and optimized a DH5α/recA+ strain for MRFα2A preparation and demonstrated minimal nuclease degradation of STC substrate in the absence of tRNA. Furthermore, additional studies using the DH5α cell extract supported previous findings that tRNA, the active component of MRFα2B, may have an active role in Mu replication. Increasing concentrations of tRNA with MRFα2A (DH5α) demonstrated a concentration dependent increase in the formation of cointegrates. With WM433/ClpX- MRFα2A extract, absence of tRNA inhibited cointegrate formation while basal levels of cointegrate formation was observed with MRFα2A prepared from DH5α. The excessive nuclease activity of WM433/ClpX- may have decreased STC substrate to the point of sub-basal levels of Mu replication. Preparation of MRFα2A was completed in a DH5α/recA+ stain.

The large-scale commercial preparation of DH5α/recA+ extract required re-optimization of the previously followed protocol. This delayed the establishment of a protocol for the purification of MRFα2A by traditional fractionation techniques. Attempts have been made to create a bioaffinity system utilizing synthetic oligonucleotides. Donor oligonucleotides were prepared containing Mu sequences promoting MuA-mediated strand transfer into target oligonucleotides. I prepared donor oligonucleotides possessing 5'-fluorescein modification as well as target oligonucleotides with 5'-biotin modification. Conditions required for optimal STC formation in this *in vitro* system being established. The STC3 nucleoprotein complex may be assembled using partially purified MRFα2A and MRFα2B. The fluorescein label would be used to follow the nucleoprotein complexes as they are isolated by streptavidin-conjugated beads which bind to the biotin-labeled target oligo. If STC3 free of nonspecifically bound proteins can be isolated, specifically bound proteins can be purified by gel electrophoresis and identified by N-terminal sequencing and mass spectrometry.

Key Research Accomplishments

- The *Escherichia coli* PriA 3' to 5' helicase activity is influenced both by fork structure and by single-strand DNA-binding protein.
- PriA recognizes and unwinds forked substrates where one or both arms are primarily duplex and requires a small (two bases or larger) single stranded gap at the fork.
- PriA is capable of translocating on either leading or lagging strand arm to unwind duplex DNA.
- Fork specific binding orients PriA helicase domain to unwind the lagging strand duplex.
- MRFα is comprised of at least three host factors, ClpX molecular chaperone, a tRNA component, and a protein component MRFα2A
- Set up a reaction system of purified proteins to assay for the remaining host protein component (MRFα2A) to catalyze Mu bacteriophage replicative transposition.
- Development and optimization of the partial purification of MRFα2A in DH5α/recA+ strain of *E. coli* .

Reportable Outcomes

Manuscripts:

Jones, J.M. and H. Nakai, *Escherichia coli PriA helicase: Synergism between fork binding and helicase activity stimulates unwinding of arrested forks.* J. Mol. Biol., 2001. **312**: p. 935-947.

Nakai, H., V. Doseeva, and J.M. Jones, *Handoff from recombinase to replisome: insights from transposition.* Proc. Natl. Acad. Sci. USA, 2001. **98**(15): p. 8247-8254.

Kim-North, S.H. and H. Nakai, *Potential Mechanisms for Linking Phage Mu Transposition with Cell Physiology*, in *Bacterial Chromosomes*, American Society of Microbiology: Washington, DC. (review will be submitted this month)

Conclusion

This project has focused on the development of a bacterial model for DNA repair by characterizing the enzymatic factors required for the initiation of DNA replication on recombination intermediates. Work completed by Dr. Jessica M. Jones demonstrated that PriA plays a critical role in the transition from recombination to DNA replication. Both fork structure and single-strand DNA binding protein influence the loading of the PriA helicase onto the Mu fork such that the lagging-strand template is preferentially unwound. This allows the loading of the major replicative DnaB helicase onto the Mu fork to initiate DNA replication. Since Dr. Jones' departure from this laboratory, the statement of work has been revised. In a year's time, I believe I have accomplished the main tasks of the revised statement of work. I have developed the skills required to carry out the reaction system for the formation of Mu cointegrate to assay for the remaining *E. coli* protein components (MRFα2A) needed for Mu DNA replication proteins. During the preparation of partially purified MRFα2A using previously described protocols, I optimized the protocol for a more suitable source of crude extract. The original WM433/ClpX$^-$ strain was found to contain excessive nuclease activity, which was disruptive for the purification of MRFα2A. Consequently we developed a new DH5/recA$^+$ strain for our source of crude extract. Finally, I have also begun to work on establishing the protocol for the isolation of the STC3 nucleoprotein complex utilizing synthetic oligonucleotides. In this past year, I feel that I have successfully established the groundwork necessary to continue the work necessary in identifying the factor(s) required to promote the assembly of replication proteins for Mu DNA synthesis.

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Handoff from recombinase to replisome: Insights from transposition

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Bacteriophage Mu replicates as a transposable element, exploiting host enzymes to promote initiation of DNA synthesis. The phage-encoded transposase MuA, assembled into an oligomeric transpososome, promotes transfer of Mu ends to target DNA, creating a fork at each end, and then remains tightly bound to both forks. In the transition to DNA synthesis, the molecular chaperone ClpX acts first to weaken the transpososome's interaction with DNA, apparently activating its function as a molecular matchmaker. This activated transpososome promotes formation of a new nucleoprotein complex (prereplisome) by yet unidentified host factors [Mu replication factors (MRFα2)], which displace the transpososome in an ATP-dependent reaction. Primosome assembly proteins PriA, PriB, DnaT, and the DnaB–DnaC complex then promote the binding of the replicative helicase DnaB on the lagging strand template of the Mu fork. PriA helicase plays an important role in opening the DNA duplex for DnaB binding, which leads to assembly of DNA polymerase III holoenzyme to form the replisome. The MRFα2 transition factors, assembled into a prereplisome, not only protect the fork from action by nonspecific host enzymes but also appear to aid in replisome assembly by helping to activate PriA's helicase activity. They consist of at least two separable components, one heat stable and the other heat labile. Although the MRFα2 components are apparently not encoded by currently known homologous recombination genes such as *recA*, *recF*, *recO*, and *recR*, they may fulfill an important function in assembling replisomes on arrested replication forks and products of homologous strand exchange.

Bacteriophage Mu's characteristics as a transposable element play a critical part in the establishment of lysogeny as well as in lytic development. On injection of phage DNA into the host cell, it is integrated at a random site of the host chromosome (1) by the phage-encoded transposition apparatus (2–4), a process that can lead to the establishment of lysogeny. Although the initial integration event is conservative (5) (i.e., not involving replication of Mu), lytic development involves many replicative transposition events (6–8) that exploit the host replication apparatus to form multiple integrated copies of Mu (8–12). Long considered a mechanism distinct from homologous recombination, Mu transposition may nevertheless have much in common with this process during the transition from strand exchange to DNA replication.

The Mu Transposition Apparatus. The establishment by Mizuuchi (8) of a crude extract system that catalyzes replicative Mu transposition has led to a detailed understanding of both the strand exchange reaction and the key steps involved in the initiation of DNA synthesis (for reviews, see refs. 13–16). This system uses a supercoiled donor substrate that bears a miniature version of the Mu genome (mini-Mu) and a target plasmid that contains no Mu DNA sequence. The strand exchange step that forms the template for Mu DNA synthesis can be catalyzed with three proteins (17): the phage-encoded transposase MuA, a second transposition protein MuB, and the host-encoded protein HU (see Fig. 1). HU aids in the assembly of MuA into an oligomeric transpososome tightly bound to both Mu ends (18–22), and the transpososome promotes integration of Mu ends into target DNA that is bound by MuB (23).

In this process, the tetrameric core of the transpososome (24–26) produces a nick at each Mu end (Fig. 1) and promotes the transfer of the resulting 3′-OH ends to target DNA (19, 21, 27). The resulting strand exchange product (28) has at each Mu end a forked structure that can become the initiation site for Mu DNA synthesis. Host replication proteins will initiate semidiscontinuous DNA synthesis at one of these forks to duplicate Mu DNA (Fig. 1) and form the final cointegrate product (12, 29). However, the transpososome remains very tightly bound to both ends after strand exchange has been completed (21). Although this transpososome appears to pose an impediment to DNA replication, it plays an important role in promoting transition to DNA synthesis (11, 30).

Host Factors Involved in Mu DNA Replication. Before the development of an *in vitro* Mu transposition system, *Escherichia coli* functions found to be required for bacteriophage Mu DNA replication included *dnaE*, *dnaX*, *dnaB*, *dnaC*, *dnaG*, *gyrA*, and *gyrB* (9, 10, 31, 32). The *dnaE* and *dnaX* genes encode subunits of the DNA polymerase (pol) III holoenzyme (33–36), the replicase at the replication fork. The DnaB protein is the major helicase at the fork, translocating 5′ to 3′ along the lagging strand template to unwind the helix for the propagating fork (37), and it can attract primase (38), encoded by *dnaG* (39), for initiation of lagging strand synthesis. DnaC protein forms a 1:1 complex with DnaB (40–42), acting as a molecular matchmaker (43) to promote loading of DnaB onto the replication fork. The *gyrA* and *gyrB* genes encode the two subunits of DNA gyrase (44–46), and this requirement may in part reflect the need for a supercoiled donor substrate for the strand exchange reaction (47, 48). As suggested by *in vivo* requirements for Mu DNA replication, we have found that cointegrate formation in the *in vitro* system requires DnaB–DnaC complex and the DNA polymerase III holoenzyme (11), confirming that the replisome involved in replicating the bacterial chromosome replicates Mu DNA during transposition.

Because Mu replication depends on host factors, we have been using this system to better understand the host apparatus needed

This paper results from the National Academy of Sciences colloquium, "Links Between Recombination and Replication: Vital Roles of Recombination," held November 10–12, 2000, in Irvine, CA.

Abbreviations: MRF, Mu replication factors; pol, polymerase; STC, strand transfer complex; DSS, disuccinimidyl suberate.

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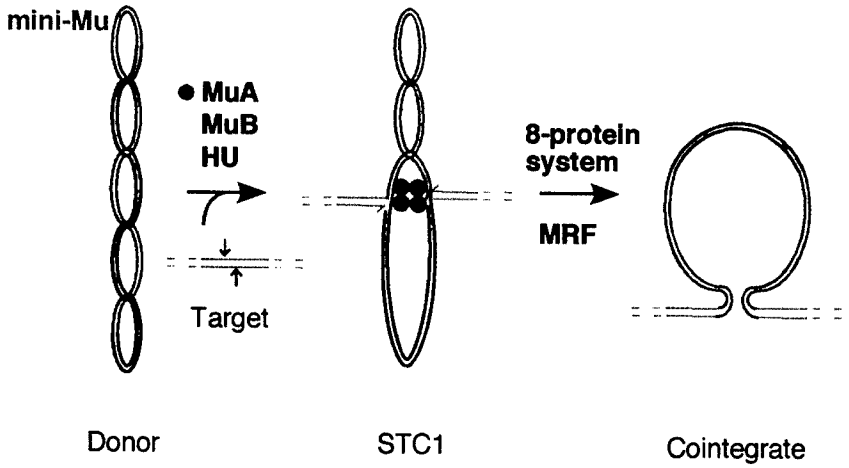

Fig. 1. Replication of Mu by transposition. In the first stage, the phage-encoded transposition proteins aided by the histone-like protein HU promote transfer of 3′-OH ends of miniMu (red) to each strand of target DNA (green). Two sites, 5 bp apart on target DNA, that will be subjected to a nucleophilic attack by each Mu end are indicated by arrows. Strand exchange produces a fork at each Mu end, the target providing 3′-OH ends (indicated by half arrows) that can potentially serve a primers for leading strand synthesis. MuA transposase, which has been assembled into an oligomeric transpososome, remains tightly bound to both Mu ends in the strand exchange product (strand transfer complex, STC1). Host factors then initiate Mu DNA synthesis from one end to duplicate Mu and form the final cointegrate product. The DNA synthesis phase was initially reconstituted in an eight-protein system supplemented with partially purified host factors (MRF), as described in the text.

to make the transition from recombination to replication. The studies were undertaken on the basis of the rationale that the Mu transposition system may be invaluable not only for dissecting the transition process in transposition but also for identifying cellular factors that play crucial roles in linking homologous recombination and replication. We initially set up a system of eight purified host proteins to identify host factors needed to initiate DNA synthesis on the product of Mu strand exchange. This system includes the DNA pol III holoenzyme, DnaB helicase, DnaC protein, primase, and DNA gyrase, the factors originally implicated in Mu replication *in vivo*. It also includes the single strand-binding protein, which would be needed at a propagating fork, as well as DNA pol I and DNA ligase, which would be needed for removing RNA primers from lagging strand synthesis and for forming covalently closed circular cointegrates, respectively. The template used for this reaction is the Mu strand exchange product formed by MuA, MuB, and HU with supercoiled donor and target substrates, the transpososome remaining very tightly bound to the two forks of this template [Fig. 1; strand transfer complex 1 (STC1)]. MuB and HU have also been found to be loosely bound to this template (49); however, we have been able to strip these proteins off STC1 without producing any apparent changes in the way it is replicated (11). Not surprisingly, the eight-protein system is not sufficient to catalyze any amount of Mu DNA synthesis on STC1. However, if the transpososome is removed from the STC1 template by phenol extraction, some of the replication proteins could gain access to the fork. On the deproteinized template, DNA pol I catalyzes limited strand displacement synthesis (11, 50). The deproteinized template can also be converted to a cointegrate in the reaction system even when DnaB is absent, provided that the DNA pol III holoenzyme preparation also contains helicase II (J.M.J. and H.N., unpublished results).

STC1 is converted to a cointegrate if the eight-protein system is supplemented with a partially purified host enzyme fraction [Mu replication factors (MRF)] (11). No cointegrates are formed when DNA pol III holoenzyme, DnaB helicase, or DnaC protein is omitted from the reaction system. If any one of these replication proteins or MRF is missing from the reaction system, we are not able to detect DnaB-independent cointegrate formation or the low levels of strand displacement DNA synthesis that can be catalyzed by DNA pol I on the deproteinized template. This indicates that the transpososome bound to the template imposes a strict requirement for both MRF and the specific replication proteins for initiation of Mu DNA synthesis. MRF was originally separated further into two fractions, MRFα and MRFβ (see Fig. 2), which can be functionally distinguished (30). MRFα removes the transpososome in an ATP-dependent reaction, and when the resulting template is isolated free of unbound proteins by gel filtration, it is converted to a cointegrate in the presence of MRFβ and the eight-protein system. MRFβ and the specific replication proteins are essential for converting this isolated template to cointegrate; however, if this template is stripped of bound proteins by phenol extraction, these factors are no longer essential for Mu DNA synthesis (30). This result implies that a new nucleoprotein complex (a prereplisome) takes the place of the transpososome and imposes specific requirements for MRFβ, DnaB, DnaC, and DNA pol III holoenzyme.

MRFα and MRFβ have each been found to consist of multiple components (see Fig. 2). The MRFα group is made up of the molecular chaperone ClpX and yet unidentified factors (MRFα2), and MRFβ is composed of primosomal constituents PriA, PriB, and DnaT (12). ClpX can play a distinct role at two different stages of the Mu life cycle (51). Together with the ClpP protein, it constitutes a chaperone-linked protease (52, 53) that can degrade the Mu immunity repressor (51, 54, 55). This process leads to derepression of Mu transposition, promoting exit out of lysogeny and induction of lytic development. ClpX, but not the protease component ClpP, is also required for Mu DNA replication *in vivo* (51), and as discussed below, it is one of the factors needed to promote transition from transpososome to replisome. The critical function of MRFβ factors *in vivo* was confirmed by the demonstration that Mu cannot undergo lytic development and cannot be replicated by transposition in a *priA* knockout mutant (12). In addition, a *dnaT* knockout mutant has recently been constructed, and it does not support Mu development (S. J. Sandler, personal communication). Although *priB* knockout mutants do support Mu development, this is consistent with the finding that *priB* has an essential cellular function that is redundant with *priC* (56). PriA, PriB, PriC, and DnaT proteins were first characterized as primosomal components needed to prime complementary DNA synthesis on single-stranded phage ϕX174 DNA (57, 58). The role of these proteins in initiating Mu DNA synthesis was consistent with their function in homologous

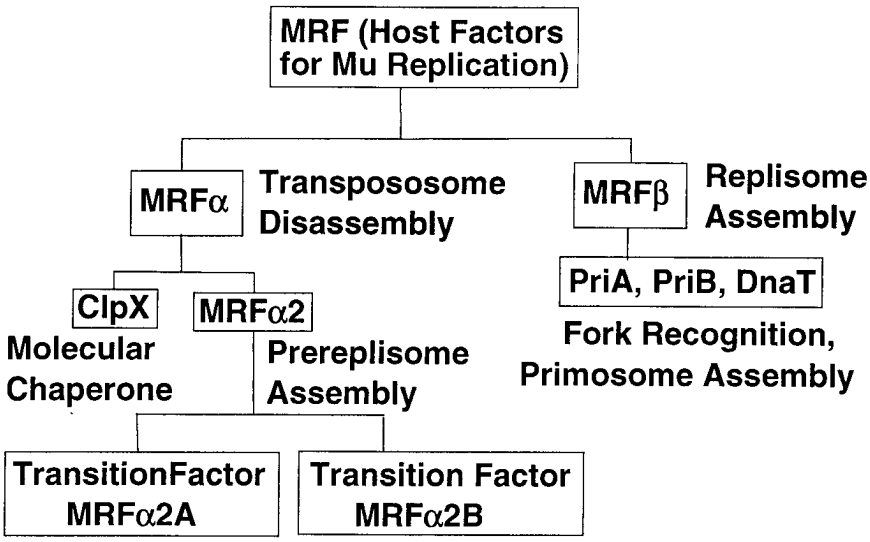

Fig. 2. Components of the MRF. MRF was originally identified as host factors needed in addition to the eight-protein system to convert STC1 to cointegrates. Resolution of MRF into enzyme fractions distinguishable by function and into pure components (ClpX, PriA, PriB, and DnaT) is indicated.

recombination envisioned by Kogoma (59, 60). He hypothesized that these primosomal components promote assembly of a replisome at the site of homologous strand exchange. Biochemical evidence that these proteins can direct replisome assembly at D-loops (61) as well as other branched structures such as the Mu fork has supported their role in initiation of DNA synthesis on recombination intermediates and in the restart of arrested replication forks (62).

Role of the Transpososome in Mu DNA Replication. A major strength of the *in vitro* Mu transposition system is that its essential components include the host factors required for Mu replication *in vivo* and that alternate pathways that initiate Mu DNA synthesis without one of these factors are prevented. As discussed so far, the transpososome plays a critical role in maintaining specificity for host factors involved in Mu DNA replication. When the transpososome is removed by phenol extraction, initiation of Mu DNA synthesis without MRF, DnaB, DnaC, or even DNA pol III holoenzyme can be detected (11, 30, 50, 63). The critical step promoted by the transpososome is the assembly of the prereplisome with MRFα2 at the Mu forks.

In the transition to DNA synthesis (Fig. 3), the molecular chaperone catalyzes the first step by acting on the transpososome in an ATP-dependent process (63, 64). Levchenko *et al.* (64) demonstrated that ClpX can cause the transpososome to disassemble, MuA dissociating from DNA in monomeric form. The released MuA could catalyze strand exchange, indicating that the cycle of transpososome assembly and ClpX-promoted disassembly produced no apparent alteration of MuA. These results indicate that ClpX can promote changes in transpososome conformation, altering MuA quaternary interactions and its interaction with DNA.

Although we also found that ClpX acts on the transpososome for the transition to DNA synthesis, our studies indicated that the transpososome must remain bound to DNA to promote the transition to DNA synthesis (63). When STC1 (the template with bound transpososome) was treated with ClpX under reaction conditions used for the *in vitro* Mu replication system, the resulting nucleoprotein complex (STC2) could be isolated free of unbound proteins (including ClpX) and then converted to a cointegrate in the eight-protein system supplemented with MRFα2 (no ClpX) and MRFβ. In isolated STC2, oligomeric MuA still holds the two Mu ends together in a synaptic complex (Fig. 3*B*). Initiation of Mu DNA synthesis strictly required MRFα2, MRFβ (or PriA, PriB, and DnaT), DnaB, DnaC, and the DNA pol III holoenzyme. If any one of these components was missing, not even partial replication of Mu DNA could be detected. However, if the transpososome was removed from DNA by phenol extraction or by high ionic strength as described below, initiation of Mu DNA synthesis did not absolutely depend on each of these components.

Even though the transpososome in STC2 remains bound to the two Mu ends, this complex is not as stable as the transpososome in STC1 (63). Intact STC2 could be isolated free of unbound proteins by gel filtration in the presence of 60 mM KCl or 200 mM potassium glutamate, ionic conditions used in the *in vitro* Mu replication system. Under conditions of higher ionic strength (300 mM NaCl), MuA dissociated from DNA. Levchenko *et al.* (64) had used such conditions to separate DNA from released MuA. This suggested to us that an intact STC2 transpososome can be isolated after ClpX treatment, so long as it is kept under conditions of lower ionic strength, which is required for the Mu replication system, and that this transpososome dissociates under conditions of higher ionic strength.

Crosslinking analysis confirmed that the oligomeric structure of the STC2 transpososome can be disrupted at 300 mM NaCl (Fig. 4). The oligomeric nature of the transpososome was originally established by chemical crosslinking (25, 27). MuA in solution is a monomer (65), and intermolecular crosslinking between MuA monomers does not readily occur. But once assembled into a transpososome, MuA protomers are crosslinked to form tetramers and even higher-order oligomers. Because the STC1 transpososome is extremely stable and remains intact even at 2 M NaCl (21), MuA protomers in the transpososome have readily been crosslinked at 0.5 M NaCl (25, 27). We performed crosslinking analysis at conditions of lower ionic strength (60 mM KCl) that allow isolation of intact STC2. Under our reaction conditions, there was very little crosslinking of MuA monomers by disuccinimidyl suberate (DSS), even when MuA was added to donor DNA without HU (conditions that do not allow the transpososome to be assembled; Fig. 4, lane 1). When STC1 isolated free of unbound proteins was treated with DSS, MuA was crosslinked to a ladder of dimers, trimers, tetramers, and higher order oligomers (lane 3). In previous studies (25, 27), the transpososome was crosslinked predominantly to tetramers by using dithio-*bis*(succinimidyl propionate),

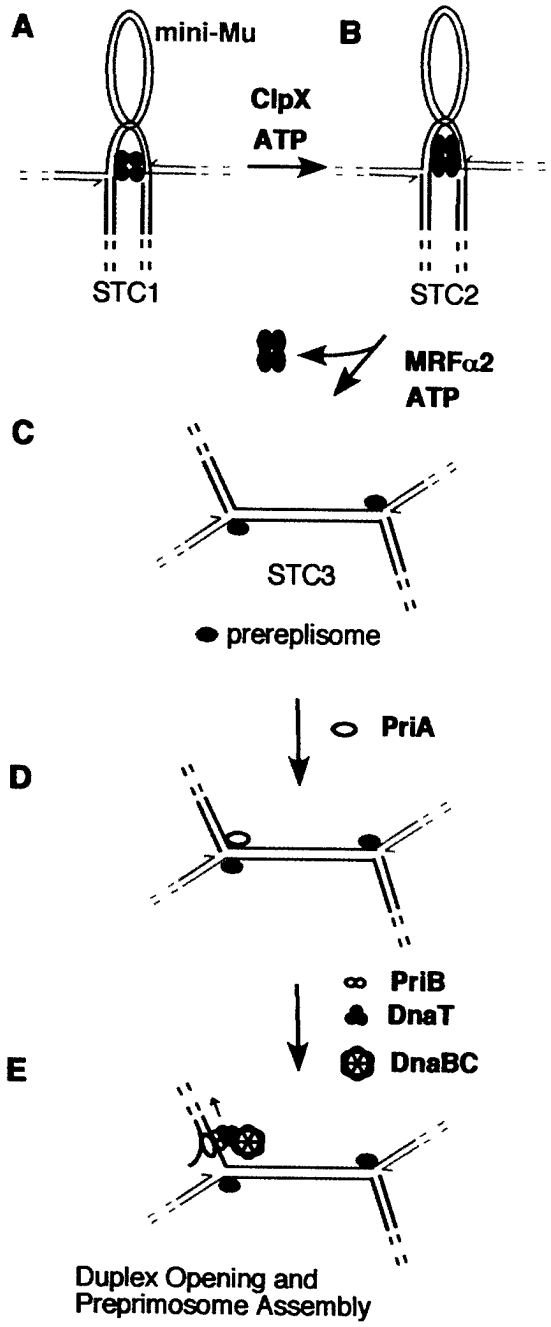

Fig. 3. Transition from transpososome to replisome. The molecular chaperone ClpX converts STC1 (*A*) to STC2 (*B*), altering the conformation of the transpososome. MRFα2 then displaces the transpososome to assemble the prereplisome at the Mu forks, forming STC3 (*C*). PriA binds to the forked DNA structure created by strand exchange (*D*) and begins the process of assembling a replisome at one Mu end. The mechanism that determines which Mu end is used to initiate DNA synthesis is not yet clear. PriA assembles a preprimosome complex by recruiting PriB, DnaT, and the DnaB-DnaC complex (*E*). In this process, DnaB must be bound to single-stranded lagging strand template. To create this binding site, PriA unwinds duplex DNA by translocating 3′ to 5′ along this template. Once bound to DNA, DnaB attracts primase to form a primosome, which catalyzes primer synthesis for lagging strand synthesis, and DnaB promotes binding of the DNA pol III holoenzyme to complete replisome assembly.

which can be cleaved by using a reducing agent. Because we used reaction conditions that largely reflect optimal conditions for Mu transposition and replication *in vitro*, we used the crosslinking agent DSS, which cannot be cleaved, and these conditions

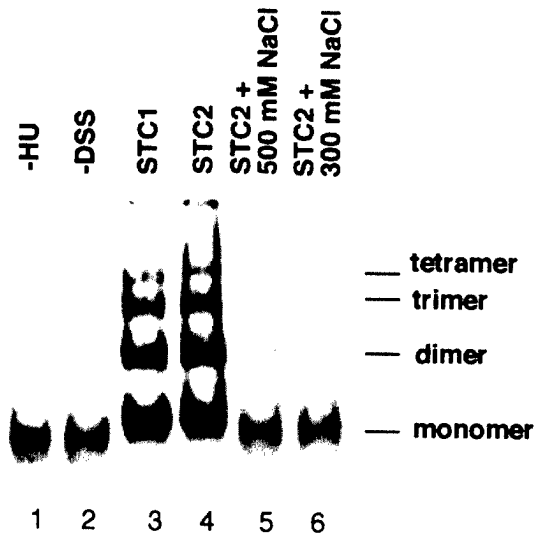

Fig. 4. Fragile property of the STC2 transpososome. Formation of STC1, its conversion to STC2, crosslinking of the transpososome with DSS, and detection of crosslinked MuA by Western blot analysis was conducted as previously described (63). Lane 1: As control, the strand exchange reaction mixture was incubated without HU protein, conditions which do not permit transpososome assembly, and then MuA was subjected to crosslinking with DSS. Lanes 2–6: STC1 was isolated free of unbound proteins by filtration through a Bio-Gel (Bio-Rad) A-15 m column equilibrated with 25 mM Hepes-KOH (pH 7.5), 12 mM magnesium acetate, and 60 mM KCl. Isolated complexes were incubated at 37°C for 30 min in the presence of ATP, ClpX being included for conversion to STC2. The reaction mixture was adjusted to 300 or 500 mM NaCl, as indicated, and allowed to stand at room temperature for 15 min before addition of DSS. For lane 2, STC1 was not subjected to DSS treatment as control.

yielded somewhat less efficient crosslinking than in previous analysis. The pattern of MuA crosslinking was not apparently changed when the isolated STC1 was converted to STC2 with ClpX (lane 4). Although no major differences in MuA crosslinking between STC1 and STC2 can be discerned, subtle changes in crosslinking between MuA protomers cannot be ruled out. Treatment of STC2 at 0.3–0.5 M NaCl resulted in the loss of MuA crosslinking, indicating that the transpososome was disassembled back to monomers (lanes 5 and 6). The resulting template isolated free of unbound proteins has all of the characteristics of the deproteinized strand exchange product: the requirement for the specific host factors in initiating Mu DNA synthesis is lost (63).

The MRFα2 Transition Factors. Although the dissociation of the STC2 transpososome at 300 mM NaCl results in loss of the requirement for the specific host factors, disassembly of this transpososome by MRFα2 forms a template that maintains this specificity (63). MRFα2 is able to disassemble the transpososome from isolated STC2 but not from STC1. This is an ATP-dependent process that results in the release of the transpososome in oligomeric form, indicated by our ability to crosslink released MuA after it has been separated from DNA by gel filtration. Whereas the STC2 transpososome can be disassembled from DNA and dissociated to monomers at 300 mM NaCl or higher, it is not yet clear how the oligomeric transpososome is dissociated from the Mu ends by MRFα2. The transpososome may simply be displaced by assembly of MRFα2 components at the Mu ends and may be fully capable of rebinding to Mu ends. Alternatively, the dissociated transpososome may be bound to an MRFα2 component that does not remain bound to the template, or it may assume an inactive

conformation such that it may no longer bind to Mu ends. Once MuA is displaced from the template, a new nucleoprotein complex (STC3) is apparently formed. STC3 isolated free of MuA retains the strict requirement for MRFβ, DnaB, DnaC, and DNA pol III holoenzyme for its conversion to a cointegrate (30, 63). The importance of proteins bound to STC3 is indicated by the loss of host factor specificity when they are removed from the template by phenol extraction. Because components of the replisome will assemble on STC3, we have referred to this nucleoprotein complex as a prereplisome that prepares the template for replisome assembly. And because we have not been able to form STC3 by incubating MRFα with the deproteinized template, we conclude that the STC2 transpososome plays a crucial role in promoting assembly of the prereplisome.

The transpososome's role in maintaining specificity for host factors is therefore associated with promoting prereplisome assembly. ClpX apparently activates a molecular matchmaker function of the transpososome (63). The STC2 transpososome allows assembly of a prereplisome in an ATP-dependent process at the Mu forks, the transpososome being displaced from DNA in the process. The interaction between prereplisome components and the Mu forks is apparently not established without the help of the transpososome. Although the transpososome remains stably bound to the Mu ends after ClpX treatment under reaction conditions used to catalyze Mu DNA replication, the presence of 300 mM NaCl or higher causes the dissociation of MuA as monomers and thus prevents prereplisome assembly. Formation of the prereplisome may commit the template to be replicated by allowing only the specific host proteins PriA, PriB, DnaT, DnaB–DnaC complex, and DNA pol III holoenzyme to gain access to the fork. If the transpososome disassembles without promoting prereplisome assembly, other cellular enzymes could compete for access to the Mu fork. The deproteinized strand transfer product can be converted to a cointegrate when introduced into a crude extract (28), but as we have discussed, this template can be converted to a cointegrate by a pathway not dependent on the specific replication factors. Thus, significant levels of alternate and aberrant products such as partially replicated and degraded templates can also accumulate.

The protein content and organization of the prereplisome complex are not yet clear. Although two identical prereplisomes are shown to be assembled at the two Mu forks in Fig. 3*C*, it is also possible that distinct complexes are assembled at these forks. In induced lysogens, replication of full-length (37-kb) Mu DNA proceeds semidiscontinuously from one end to the other, and Mu DNA synthesis initiates 80–90% of the time from the Mu left end (29, 66–68). DNA synthesis in the reconstituted system also initiates at one end and proceeds semidiscontinuously to the other end, and preference for the Mu left end can be detected (12). These results indicate that PriA promotes assembly of a replisome predominantly at one Mu end, preferentially the left end, and not both (Fig. 3*D*). One possible mechanism for the choice of Mu ends used for replisome assembly would be the asymmetric assembly of nucleoprotein complexes at the two Mu ends during transition to STC3. The two ends are composed of different sequences, and the transpososome is bound differently to each end (25, 27). It is therefore possible that the transpososome promotes asymmetric prereplisome assembly such that replisome assembly at the left end is favored.

We are currently purifying MRFα2 to identify the remaining host factors needed to replicate Mu DNA during transposition. We have so far determined that MRFα2 can be resolved into two components (MRFα2A and MRFα2B). MRFα2 activity is assayed by using a reaction system that includes STC1 as template, molecular chaperone ClpX, and purified replication proteins (the original eight-protein system plus PriA, PriB, PriC, and DnaT). This assay system cannot promote cointegrate formation unless supplemented with both MRFα2 components (Fig. 5, compare lane 6 with lanes 4 and 5). The conclusion that these are two distinct factors is confirmed by the finding that one is heat labile, whereas the other is heat stable. MRFα2A is readily inactivated by heating at 65°C for 10 min (lane 7), whereas MRFα2B remains active even after heating at 100°C for 10 min (lane 8).

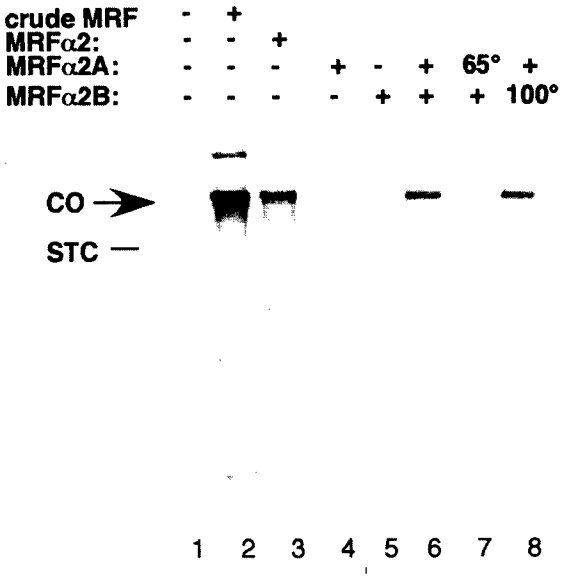

Fig. 5. MRFα2 consists of at least two distinct components. The reconstituted Mu DNA replication reaction (50 μl) with [α-^{32}P]dNTPs was assembled with ClpX, the 12-protein system, and the indicated MRFα2 components, and products were resolved by alkaline agarose electrophoresis as previously described (75). Crude MRF (fraction II) (30) and MRFα2 (fraction III) (63) were prepared as described. Resolution of MRFα2 into two components, MRFα2A and MRFα2B, will be described in a future publication (V.D. and H.N., unpublished work). Approximately 10 units (63) of the indicated MRFα2 components were added. Where indicated, MRFα2A and MRFα2B were heated at 65 and 100°C, respectively, for 10 min. In the reaction catalyzed with crude MRF, greater than 95% of STC1 was converted to cointegrate. When MRFα2 was supplied as two components, typically 50–95% of STC1 was converted to a cointegrate. CO, position of the cointegrate; STC, position of the strand exchange product (not radiolabeled and therefore not visible).

Genetic analysis has not yet suggested the possible identity of MRFα2 components. The critical role PriA plays in both homologous recombination and Mu replication has suggested that Mu may exploit host homologous recombination functions for the transition from recombination to replication. We have examined a number of homologous recombination functions, including those that play a critical role in restart of chromosomal replication (for reviews, see refs. 62 and 69–72), for a possible role in Mu replication. So far, we have not found mutants that are as defective in Mu development as the *priA* and *dnaT* knockout mutants. We have examined *recA*, *recF*, *recO*, *recR*, *recJ*, *recG*, and *ruvA* mutants, most of which have knockout mutations (exceptions are *recF143* and *recF4101* strains provided to us by S. Sandler, University of Massachusetts, Amherst, MA), and all were able to support Mu lytic development. S. Lamrani and G. Maenhaut-Michel (personal communication) have also found that knockout mutations in the following genes still allow Mu lytic development to proceed: *recA*, *recB*, *recD*, *recG*, *ruvA*, *ruvC*, *ruvABC*, and *rusA*. We cannot rule out the unlikely possibility that more than one host factor can provide the same MRFα2 function and that one of these recombination genes provides such a redundant function. On the other hand, MRFα2 components may turn out to be factors not yet implicated in any step in recombination or replication. Just as MRFα2 components may act at a stage when the transpososome function is

being completed and the replisome function is about to begin, they may act at a corresponding stage in cellular recombination-dependent replication, having a function distinct from that of the currently known homologous recombination proteins.

Role of PriA's Helicase Activity. The involvement of host factors such as PriA in Mu DNA replication has helped us better understand their role in cellular chromosome replication and recombination. For example, the essential role of PriA in Mu DNA replication *in vitro* and *in vivo* has demonstrated the critical function it can play in assembling replisomes on recombination intermediates (12). In addition, PriA's 3′ to 5′ helicase activity (73, 74) has been found to play an important role in initiating Mu DNA replication (75). PriA mutants defective in helicase activity such as PriA K230R are proficient in assembling a primosome on the single-stranded template of phage ϕX174, and they are able to reverse characteristics of slow growth, low viability, and filamentous morphology (76) characteristic of *priA* knockout strains (77, 78). Although the cellular phenotype of *priA* helicase mutants suggests no apparent function for the helicase activity, studies of the role of PriA helicase in Mu DNA replication indicate a general function in duplex opening for replisome assembly (13, 75).

Mutants expressing PriA K230R support Mu DNA replication at greatly reduced rates (less than 20% the rate of wild-type cells). Infected cells exhibit delayed lysis and a reduced burst, and when Mu is plated with these mutants, minute plaques are formed. In the reconstituted system, which consists of the purified protein system supplemented with partially purified MRFα2, little to no cointegrates can be formed if PriA K230R is used instead of wild-type PriA. Unlike the potential replication fork at a D-loop created by homologous strand exchange, the Mu fork created by the transposase has no single-stranded DNA on the lagging strand arm of the fork to load the DnaB helicase: there is a gap of only five bases on the leading strand side of the fork. To assemble a replisome at the fork, DnaB must be bound to the lagging strand template of the fork, and it occupies 20 nucleotides of single-stranded DNA (79). PriA binds to the forked DNA structures found at D-loops, arrested replication forks, and the Mu fork (75, 80, 81), and then PriA can translocate 3′ to 5′ along the lagging strand template as it promotes preprimosome assembly with PriB, DnaT, and the DnaB-DnaC complex (75) (Fig. 3*E*). Translocation of PriA along that template is tightly coupled to the binding of DnaB to the same DNA strand. Thus, PriA helicase can function to create the single-stranded template needed for primosome assembly when there is an insufficient amount needed to load DnaB.

Although inactivation of PriA helicase greatly reduces the rate of Mu DNA replication *in vivo*, it does not eliminate Mu DNA replication entirely. This is most likely because of the action of other host factors such as other helicases and exonucleases that may also serve to create a duplex opening on the lagging strand side of the fork. Even though Mu DNA replication is catalyzed poorly in the reconstituted system by using PriA K230R, addition of a crude host enzyme fraction can complement this deficiency to promote higher levels of cointegrate formation (75). For DNA synthesis at a D-loop, PriA helicase activity may not be required because the template already has a duplex opening for loading DnaB. But other pathways for restarting DNA replication, such as the regression of the replication fork, could very well require duplex opening (13). Even if such pathways requiring duplex opening are critical for cell viability, mutations that inactivate PriA helicase may exhibit no severe phenotype because other host enzymes may also carry out this function. At least in the case of Mu, however, duplex opening by other factors is inefficient because helicase deficiency impairs Mu replication *in vivo*. For its role in cellular DNA recombination and replication, PriA may require its helicase activity to function optimally, and this may be reflected by the observation that the helicase motif of *priA* genes identified in various species is highly conserved (13).

In the initiation of chromosomal replication at the bacterial origin *oriC*, a critical point of regulation is duplex opening catalyzed by the initiator protein DnaA (82). At the Mu fork, the prereplisome may influence whether PriA helicase can open the DNA duplex. The deproteinized strand exchange product is not so readily converted to a cointegrate in the reconstituted system (no helicase II present), especially when DNA pol I is omitted so that limited strand displacement synthesis, which can create a duplex opening, is not catalyzed. At optimal levels, only about 30–40% of the deproteinized strand exchange products are converted to cointegrates in the absence of DNA pol I (12); greater than 95% of STC1 can be converted to cointegrate in the reconstituted system. Examining PriA helicase activity by using oligonucleotide substrates, we have found that oligonucleotides with the structure of the Mu fork are not good substrates for PriA helicase even though they are bound with high affinity by PriA (ref. 75; J. M. Jones and H.N., unpublished work). Two alterations of the Mu fork substrate increase unwinding of the duplex lagging strand arm. Significant amounts of unwinding can be detected if the leading strand arm of the fork is rendered single stranded. A similar amount of unwinding can be detected if a five-base gap is introduced at the fork on the lagging strand arm. The two modifications together increase unwinding $\approx$10-fold over the DNA substrate that has only one of the modifications.

Nurse *et al.* (80) have characterized two distinct modes of PriA binding to DNA. One mode is reflected by binding of PriA to duplexes with 3′ single strand extensions. This is thought to reflect recognition of DNA by the helicase domain. In the second mode, PriA binds to forked substrates, recognizing bent DNA in three-arm junctions. Our recent results (J. M. Jones and H.N., unpublished work) indicate that the fork-specific binding mode leads to translocation of PriA 3′ to 5′ along the lagging strand template, suggesting that this mode of binding orients the helicase domain to bind to this strand. Alterations of the Mu fork that promote PriA helicase action facilitate access of the single strand to which the helicase domain binds. The assembly of the prereplisome at the Mu fork may hold the fork in a conformation that allows activation of the PriA helicase without these alterations.

Potential Role of Transition Mechanisms in Cellular DNA Replication. The transition mechanisms involved in Mu DNA replication promote the assembly of a replisome at the Mu fork with specific host factors, apparently excluding access of the fork to nonspecific host enzymes that can lead to inefficient Mu replication or that may damage the template. Such mechanisms may also play a very important role in cellular DNA replication linked to homologous recombination. MRFα potentially consists of two types of components. One type comprises host factors that normally do not function in cellular recombination-dependent replication. That is, the transposase has evolved to exploit these factors to promote transition to DNA replication. For example, the molecular chaperone ClpX may not play any function in linking cellular recombination and replication; its role in linking recombination and replication may be limited to activating the transposase's molecular matchmaker function. A second type of MRFα component may be factors that have evolved to function in the transition between recombination and replication, preparing DNA recombination intermediates for replisome assembly. Components of the prereplisome in STC3 most likely belong to this class. Presumably, they did not evolve only to promote the transition process in transposition.

Assembly of a prereplisome on a template created by homologous recombination proteins may commit the pathway to the assembly of a replisome and the establishment of a replication fork. The prereplisome assembly step may distinguish this path-

way from other processes associated with homologous strand exchange such as double-strand break repair or replication-dependent recombination (69), which may require only a limited amount of DNA synthesis linked to recombination and not a highly processive replisome needed for chromosomal replication. In pathways that repair DNA lesions at an arrested replication fork, the prereplisome may play a critical function to assure re-establishment of the replication fork by requiring that DnaB and DNA pol III holoenzyme be engaged on that template.

If the prereplisome components evolved to promote transition from homologous recombination to replication, what processes might be involved in assembling the prereplisome at the site of homologous strand exchange? In transposition, the transpososome may aid in prereplisome assembly in one of two ways. First, the transpososome may interact specifically with prereplisome components to recruit them to the Mu fork. Such a function may be assumed by one of the homologous recombination proteins such as RecA at a D-loop. Alternatively, the transpososome may hold the DNA together in a conformation that mimics a structure created by homologous recombination proteins as they promote restart of chromosomal replication. This type of strategy is used in recruiting PriA, which binds to the branched DNA structure created by Mu strand exchange (75), a structure resembling D-loops and arrested forks. The STC2 transpososome still holds the two Mu forks together in a synaptic complex (63), but unlike the STC1 transpososome, this complex is fragile. ClpX's role in weakening the transpososome's interaction with DNA (63, 64) may be the key feature of activating the apparent molecular matchmaker function of the transpososome. As the prereplisome assembles on the DNA structure maintained on STC2, the transpososome disassembles, completing the handoff of the Mu forks from phage-encoded transposase to host factors. How cellular recombination functions trigger prereplisome assembly may be a key to understanding what regulates replisome assembly during homologous recombination. The identification and characterization of MRFα2 transition factors promise to provide better understanding of the transition between recombination and replication for the transposable element as well as its host.

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Escherichia coli PriA Helicase: Fork Binding Orients the Helicase to Unwind the Lagging Strand Side of Arrested Replication Forks

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Escherichia coli PriA is a primosome assembly protein with 3′ to 5′ helicase activity whose apparent function is to promote resumption of DNA synthesis following replication-fork arrest. Here, we describe how initiation of helicase activity on DNA forks is influenced by both fork structure and by single-strand DNA-binding protein. PriA could recognize and unwind forked substrates where one or both arms were primarily duplex, and PriA required a small (two bases or larger) single-stranded gap at the fork in order to initiate unwinding. The helicase was most active on substrates with a duplex lagging-strand arm and a single-stranded leading-strand arm. On this substrate, PriA was capable of translocating on either the leading or lagging strands to unwind the duplex ahead of the fork or the lagging-strand duplex, respectively. Fork-specific binding apparently orients the helicase domain to unwind the lagging-strand duplex. Binding of single-strand-binding protein to forked templates could inhibit unwinding of the duplex ahead of the fork but not unwinding of the lagging-strand duplex or translocation on the lagging-strand template. While single-strand-binding protein could inhibit binding of PriA to the minimal, unforked DNA substrates, it could not inhibit PriA binding to forked substrates. In the cell, single-strand-binding protein and fork structure may direct PriA helicase to translocate along the lagging-strand template of forked structures such that the primosome is specifically assembled on that DNA strand.

Keywords: PriA helicase; single-strand-binding protein; replication-fork arrest; replication restart; linkage of recombination and replication

Introduction

The bacterial primosome assembly protein and helicase PriA was originally characterized based on its requirement for bacteriophage φX174 complementary strand synthesis. PriA binds to a unique secondary-structural element on φX(+) DNA called the primosome assembly site (PAS) and assembles additional primosome components PriB, PriC and DnaT.[1–3] The major cellular replicative helicase DnaB is then recruited from the DnaB-DnaC complex and loaded onto single-stranded DNA 3′ of the PAS.[2–4] The preprimosome complex comprising PriA, PriB, PriC, DnaT and DnaB translocates on the single-stranded template,[4,5] recruiting the DnaG primase for the synthesis of RNA primers. While the preprimosome can translocate in either direction,[5] the primary polarity of translocation is in the direction opposite that of primer synthesis,[4] suggesting that the DnaB 5′ to 3′ helicase is the dominant activity. Point mutants that inactivate PriA's 3′ to 5′ helicase activity do not disrupt preprimosome assembly on the PAS or primer synthesis on the φX(+) template.[6–8]

PriA is now known to play vital roles in bacterial metabolism, with inactivating mutations in *Escherichia coli* characterized by severely reduced viability and homologous recombination as well as

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Abbreviations used: PAS, primosome assembly site; D-loop, displacement loop homologous recombination intermediate; A-fork, arrested replication fork; SSB, single-strand-binding protein; bp, base-pair; SYN, synergistic; BAS, basal; MRFα_2, Mu replication factor α_2; PNK, polynucleotide kinase.

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slow growth.[9–11] Current models suggest that this phenotype results from the requirement for PriA in the initiation of replication on forked DNA structures such as homologous recombination intermediates (D-loops) and arrested replication forks (A-forks).[12–14] To assemble a replisome on forked templates, the major helicase DnaB must be bound to the lagging-strand template of the fork such that it translocates 5′ to 3′ along that strand. This process unwinds the DNA helix for replication and attracts the primase needed to initiate lagging-strand synthesis. Assembly of a preprimosome at a fork by PriA would serve this important function of loading DnaB.

Replication forks initiated at the chromosomal origin can stall before completing replication, particularly when the cell has suffered DNA damage.[13,15–17] Replication and homologous functions that promote restart of replication are therefore vital components needed to replicate the chromosome. The A-fork structure shown in Figure 1 results when DNA polymerase encounters a blockage on the leading-strand template and lagging-strand synthesis continues for one or more additional rounds prior to the dissociation of DnaB helicase,[12,18] creating a fork with a single-stranded leading-strand arm and a primarily duplex lagging-strand arm. While this structure has only been inferred in *E. coli*, formation of such a product has been observed directly when DNA replication was reconstituted with eukaryotic cell extract on templates that have thymine dimers.[19,20] Initiation of DNA replication on DNA forks provides several possible pathways for resuming replication wherever fork arrest has occurred. If the A-fork is converted into a double-strand DNA break,[21] resection of the break and strand invasion mediated by homologous recombination proteins will create a D-loop from which replication can be re-initiated.[12,13] Under some circumstances the A-fork may be directly re-activated by PriA, allowing for the resumption of replication without the requirement for breakage and resection.[14] Re-activation of the A-fork would rely on the coupling of PriA helicase and preprimosome-assembly activities as has been demonstrated on synthetic A-forks.[22]

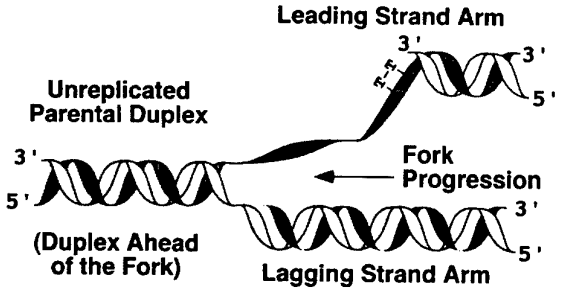


Figure 1. The arrested replication fork (A-fork). When the polymerase travelling on the leading-strand template encounters a lesion, in this case a thymine dimer, lagging-strand synthesis may continue for an additional round. This produces a fork with a primarily duplex lagging-strand arm and a single-stranded leading-strand arm.

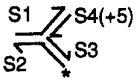
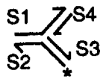
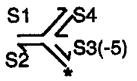
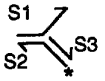
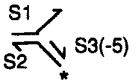
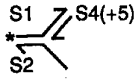
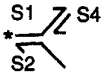
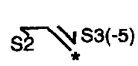
PriA has been shown to promote preprimosome assembly and DNA replication on synthetic D-loops[23,24] and is required for the initiation of replication on the forked DNA intermediate created by transposase during bacteriophage Mu transposition.[22,25] Both of these reactions are absolutely dependent on PriA's ability to assemble the preprimosome and result in the loading of the DnaB helicase on the lagging-strand template. While PriA's 3′ to 5′ helicase activity can interfere with preprimosome assembly on a D-loop,[24] it plays an important role in the initiation of DNA synthesis on the Mu fork both *in vitro* and *in vivo*.[22] Unlike the D-loop, the Mu fork includes no regions of single-stranded DNA large enough to load the DnaB helicase, which requires a duplex opening of at least 20 bases.[26] This suggests that PriA's helicase assists in preprimosome assembly only on substrates where insufficient single-stranded DNA is available for the loading of DnaB. A-forks resulting from a blockage of the DNA polymerase on the leading-strand template may also lack large regions of single-stranded DNA on the lagging-strand side (Figure 1). On synthetic forks with the structure of an A-fork, PriA unwinds the lagging-strand arm while promoting preprimosome assembly.[22]

PriA can bind to and unwind a wide variety of forked DNA substrates, with the minimum requirement for unwinding being an unforked duplex with a 3′ single-stranded extension.[22,27,28] It is likely that PriA helicase is less promiscuous in the cell. Here we report that activation of the helicase is strongly influenced by subtle aspects of substrate structure and by the presence of single-strand DNA-binding protein (SSB). SSB inhibited PriA binding to duplexes with a 3′ single-strand extensions but not to forked templates. Fork-specific binding of PriA to SSB-bound templates promoted translocation of PriA along the lagging-strand template but not on the leading-strand template. These properties may restrict PriA helicase action to the lagging-strand arm of the fork to produce the duplex openings needed for preprimosome assembly.

Results

We explored the requirements for the engagement of helicase activity using a series of forked oligonucleotide substrates. Substrate names were in keeping with a scheme developed previously.[22] The basic substrate used in these assays was substrate E[−0/−0] (Table 1, line 1), a fork with duplex arms, having no gap on either the top (leading-strand) or bottom (lagging-strand) arms. On all substrates the number or numbers in brack-

Table 1. PriA helicase activity in the absence of SSB

Substrate[a]		Total Substrate Consumed (fmol)[b]	Representative Distribution of Labeled Products (fmol)
1) E[-0/-0]		< 0.1	
2) E[-5/-0]		2.4±0.9	1.1 1.3
3) E[-5/-5]		1.1±0.9	0.5 0.3 0.3
4) C[-0]		5.8±0.9	0.3 3.1 2.4
5) C[-5]		5.0±0.6	2.6 2.3 0.1
6) D		3.7±0.5	3.7
7) F[-0]		0.3±0.0	0.3
8) F[-5]		1.1±0.3	1.1
9) W		1.3±0.3	1.3

[a] Substrates were constructed from the oligonucleotides indicated; the 5′ end-labeled oligonucleotide is indicated with an asterisk. The size of the gap at the fork is indicated in brackets for each substrate; for substrate E, the first number in brackets indicates the gap on the leading-strand (top) arm, and the second number indicates the gap on the lagging-strand (bottom) arm. The length of the duplexes are S1-S2, 30 bp; S2-S3, 70 bp, S1-S4(+5), 40 bp.

[b] Helicase assays were performed as described in Materials and Methods using 16 fmol substrate. Reactions did not include SSB. Values are the average of three or more independent trials ± standard deviation of the mean.

ets refer to the size of the gap or gaps at the fork, with the gap on the top arm listed first if two gaps are present. Substrate E[−0/0] was composed of four oligonucleotides (S1, S2, S3 and S4(+5); Figure 2), and additional substrates were constructed by shortening and/or omitting one or more of these oligonucleotides. Substrates tested included those resembling A-forks with single-stranded leading-strand arms (substrates C[−0] and C[−5]; Table 1, lines 4 and 5, respectively), those resembling the fork at a D-loop with single-stranded lagging-strand arms (substrates F[−0] and F[−5]; Table 1, lines 7 and 8, respectively), those with two single-stranded arms (substrate D; Table 1, line 6), and the minimal linear duplex substrate with a 3′ single-stranded tail (substrate W, Table 1, line 9). PriA helicase is not active on completely duplex linear substrates or linear substrates with a 5′ tail,[27] and substrates with those structures were therefore not tested here.

In these assays, PriA was incubated with the substrate for ten minutes on ice, during which time no unwinding could be detected (data not shown). Reactions were then transferred to 30 °C for 15 minutes. For reactions that included SSB, it was added to the reactions just prior to transfer to 30 °C unless otherwise indicated. Reaction products were separated on polyacrylamide gels and the amount of each labeled product was quantified by phosphorimagery of dried gels.

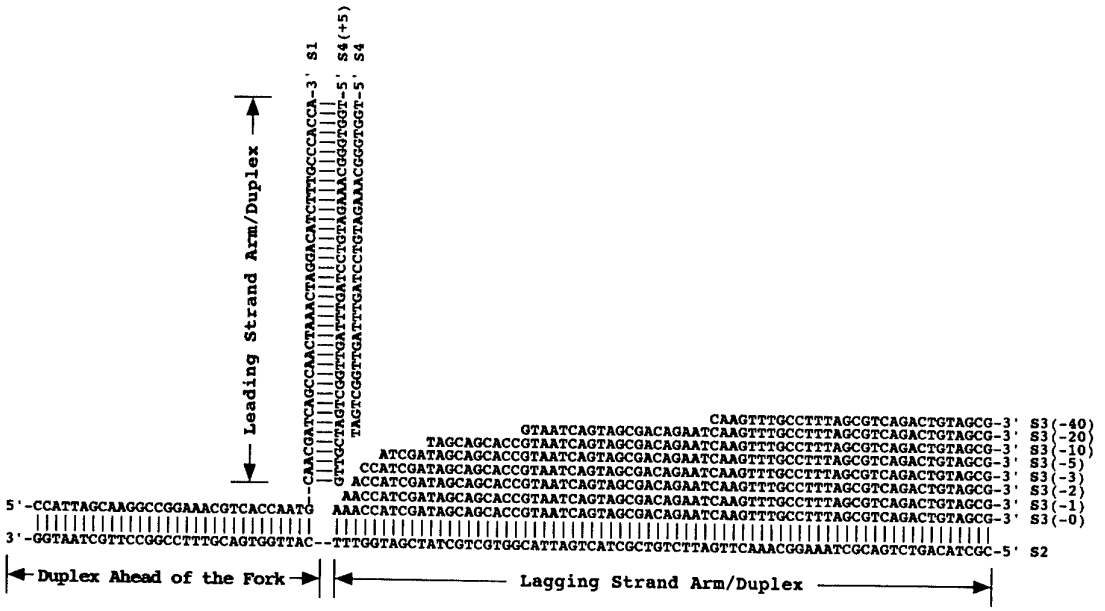


Figure 2. Oligonucleotides used for fork substrates. Nucleotide sequence, polarity and complementarity are shown. Dashes at the fork junction are for clarity and do not represent discontinuities in the oligonucleotides. Duplex nomenclature is as follows: duplex ahead of the fork, S1-S2; lagging-strand duplex, S2-S3; leading-strand duplex, S1-S4. The 3′ end of oligonucleotide S4 is analogous to the primer for leading-strand synthesis at a replication fork.

PriA helicase requires a small single-stranded gap to initiate unwinding

Helicase activity was initially examined in the absence of SSB (Table 1). We reported previously that a small gap at the fork was required for the initiation of helicase activity,[22] and that finding was examined in further detail here. PriA was completely inactive on substrate E[−0/−0], which included no gap at the fork on either arm (Table 1, line 1). Introduction of a five-base gap at the fork on the leading-strand (top) arm to create substrate E[−5/−0] stimulated unwinding of the S1-S2 duplex ahead of the fork with 2.4(±0.9) fmol out of 16 fmol total substrate being consumed (Table 1, line 2). Unwinding of the S1-S2 duplex is most likely the result of PriA binding to the leading-strand template at the fork and traveling in a 3′ to 5′ direction on that strand. Unwinding of the S2-S3 duplex alone (the lagging-strand arm) would create a product with a duplex leading-strand arm and a single-stranded lagging-strand arm (similar to substrate F[− 5]), but no products of this type were present. Single-stranded products corresponding to unwinding both the S1-S2 and S2-S3 duplexes were detected, but in this case, the unwinding of the S2-S3 duplex was most likely unwound after unwinding of the S1-S2 duplex had been initiated. For example, unwinding of the S1-S2 duplex would create a duplex with a 3′ single-stranded extension, providing a minimal helicase substrate for unwinding the S2-S3 duplex. The results indicate that PriA can only initiate unwinding on the arm that includes the small gap. Products corresponding to the unwinding of the S2-S3 duplex alone were generated when a small gap was introduced on the lagging-strand arm to create substrate E[−5/−5] (Table 1, line 3). Such products represented 30-50 % of the substrates consumed.

PriA was active on substrate C[−0], which had a single-stranded leading-strand arm and no gap at the fork on the lagging-strand arm, consuming 5.7(±0.9) fmol of substrate in the absence of SSB (Table 1, line 4). As with substrate E[−5/−0], PriA unwound primarily the S1-S2 duplex ahead of the fork on this substrate, consistent with the hypothesis that a region of single-stranded DNA is required for the initiation of unwinding. The introduction of a five-base gap at the fork on the lagging-strand arm to create substrate C[−5] permitted the unwinding of the S2-S3 duplex by PriA (Table 1, line 5); roughly half of the products detected resulted from the unwinding of the S2-S3 duplex alone. PriA had very little activity on substrate F[−0], a substrate with a single-stranded lagging-strand arm and a fully duplex leading-strand arm (Table 1, line 7), but the unwinding of the S1-S2 duplex was increased three to fourfold on substrate F[−5], which included a gap at the fork.

Only a very small single-stranded gap is required to allow PriA to initiate unwinding. Substrate C was used to explore the requirement for a gap in greater detail. In the absence of SSB, a gap as small as two bases was sufficient to permit the unwinding of the S2-S3 duplex by PriA, while a one-base gap was not (Figure 3(b), open squares). The unwinding increased for gaps of three and five bases, and continued to increase gradually as the

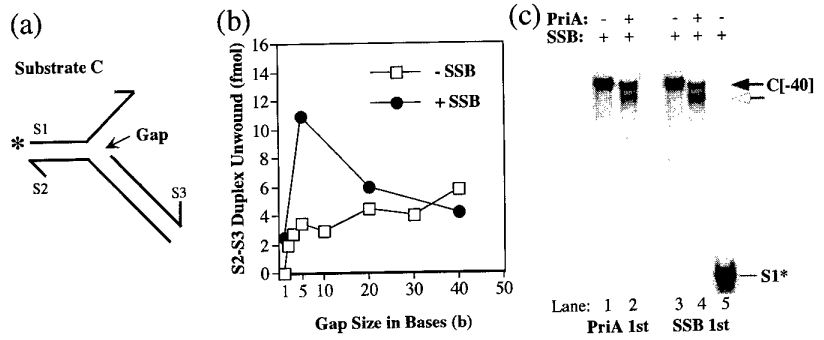


Figure 3. The effect of gap size and SSB on PriA helicase activity. (a) Substrate C, labeled (*) on the 5′ end of the S1 oligonucleotide. Substrates with gaps of increasing size on the lagging-strand at the fork were created using a series of S3 oligonucleotides that were truncated on the 5′ end. (b) Helicase assays using PriA and SSB as indicated were conducted on substrates C[−1] to C[−40] (16 fmol) as described in Materials and Methods. The unwinding of the S2-S3 duplex was quantified. The first four data points for the −SSB condition (open squares) are for substrates C[−1], C[−2], C[−3] and C[−5]. The first data point for the +SSB condition is for substrate C[−1]. (c) Helicase assays were performed on substrate C[−40] (lanes 1-4). The order of addition for PriA and SSB is indicated. Filled arrow, substrate; open arrow, product resulting from the unwinding of the S2-S3 duplex. The S1-S2 duplex is not unwound in the presence of SSB. The position of labeled S1 oligonucleotide is indicated (lane 5).

gap was expanded to 40 bases. As the gap on substrate C increased, the length of the duplex to be unwound decreased from 60 to 30 base-pairs (bp). In a variety of experiments we saw no evidence that a decrease in duplex length in this size range would significantly increase apparent helicase activity (see below and data not shown).

We reported previously that forked oligonucleotide substrates, including Mu sequences, did not require a gap at the fork for the initiation of unwinding by PriA.[22] However, those substrates included much shorter lagging-strand arms (28 *versus* 70 bp) than the structurally equivalent substrates used here. When the lagging-strand arm on the substrate including Mu sequences was lengthened to 70 bp we found that a small gap at the fork was indeed required for the initiation of helicase activity (data not shown). The length of the lagging-strand arm also distinguishes these substrates from those used by McGlynn *et al.*, who found no requirement for a gap at the fork.[27] We have not determined the mechanism by which the length of the arm affects initiation of unwinding at the fork. PriA's footprint on substrates resembling D-loops suggests that it may interact with regions up to 40 bases away from the fork junction,[23] perhaps by the wrapping of the DNA around the protein. It is possible that such interaction may influence the initiation of helicase activity.

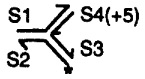
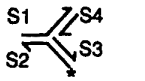
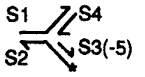
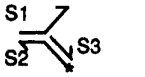
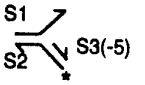
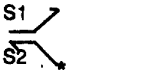
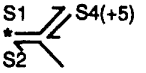
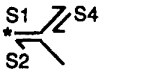
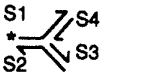
PriA can translocate on either the leading or lagging-strand templates of the fork, but not on the primer for leading-strand synthesis

PriA helicase was only active on certain strands at the fork. It could unwind the duplex ahead of the fork (S1-S2 duplex) or the lagging-strand duplex (S2-S3 duplex) depending on the substrate and reaction conditions. As is mentioned in the preceding section, unwinding of the S1-S2 duplex is likely to result from PriA translocating on the leading-strand template, and unwinding of the S2-S3 duplex is likely to result from PriA translocating on the lagging-strand template. PriA could not, however, unwind the leading-strand duplex (S1-S4 duplex). Unwinding of this duplex would occur if PriA binds to the leading-strand primer at the fork and translocates in a 3′ to 5′ direction. However, no unwinding of the S1-S4 duplex on substrates E[−5/−0] and E[−5/−5], which were radiolabeled on the S2 strand, could be detected (Tables 1 and 2, lines 2 and 3). Even when substrate E[−5/−0] was labeled on the S1 strand so that any reaction products in which the S1-S4 duplex had been unwound could be detected, no products corresponding to the unwinding of the S1-S4 duplex were detected (Table 2, line 10). Similarly, no unwinding of the S1-S4 duplex on substrates F[−0] and F[−5] was detected (Tables 1 and 2, lines 7 and 8). Thus, PriA is confined to translocate along the leading or lagging-strand template at the fork. Being fully hybridized to the template, the leading-strand primer would not have any exposed single-strand on the 3′ end at which PriA could initiate helicase action. The lack of single-stranded DNA as well as the polarity of the PriA helicase most likely accounts for its lack of activity on the leading-strand arm of the fork.

The effect of SSB on PriA helicase and fork-binding activities

SSB was not required for helicase activity on any of the forked oligonucleotide substrates. SSB substantially reduced PriA helicase activity on substrates C[−0] (cf. line 4 from Tables 1 and 2) and D (cf. line 6 from Tables 1 and 2), which are both forks with single-stranded leading-strand arms, as

Table 2. PriA helicase activity in the presence of SSB

Substrate[a]		Total Substrate Consumed (fmol)[b]	Representative Distribution of Labeled Products (fmol)		
1) E[-0/-0]		< 0.1			
2) E[-5/-0]		3.2±0.3		1.3	1.9
3) E[-5/-5]		3.5±0.8	1.0	1.3	1.2
4) C[-0]		0.9±0.0	0.3	0.3	0.3
5) C[-5]		9.0±3.0	8.0	1.0	
6) D		0.8±0.2			0.8
7) F[-0]		0.8±0.2		0.8	
8) F[-5]		1.3±0.3		1.3	
9) W		0.3±0.0			0.3
10) E[-5/-0]		1.2[c]		1.2	

[a] Substrates were constructed from the oligonucleotides indicated; the 5′ end-labeled oligonucleotide is indicated with an asterisk. The size of the gap at the fork is indicated in brackets for each substrate; for substrate E, the first number in brackets indicates the gap on the leading-strand (top) arm, and the second number indicates the gap on the lagging-strand (bottom) arm. The length of the duplexes are S1-S2, 30 bp; S2-S3, 70 bp, S1-S4(+5), 40 bp.

[b] Helicase assays were performed as described in Materials and Methods using 16 fmol substrate. All reactions included SSB. Values are the average of three or more independent trials ± standard deviation of the mean.

[c] Standard deviation not determined.

well as substrate W (cf. line 9 from Tables 1 and 2), which is the duplex with the 3′-single-strand tail. On these substrates, it is likely that PriA binds to the single-stranded DNA and translocates 3′ to 5′ along that strand to unwind the duplex. This inhibition occurred even though PriA was incubated with the substrates for ten minutes on ice prior to the addition of SSB. We did not see this inhibitory effect when the leading-strand arm of the fork included only a five-base gap (e.g. Table 2, lines 2 and 8), which is insufficient for stable SSB binding.[29] This suggests that SSB can interfere with the initiation of PriA translocation on single-stranded DNA that is long enough to be bound stably by SSB.

Although SSB inhibited unwinding of substrate D (Figure 4(a), cf. lanes 2-4 with lanes 6-8), SSB did not inhibit PriA binding to substrate D (Figure 4(b)). In band-shift assays PriA alone produced a discrete band shift with substrate D (Figure 4(b), lanes 2-3) as did SSB alone (Figure 4(b), lane 4). SSB almost completely inhib-

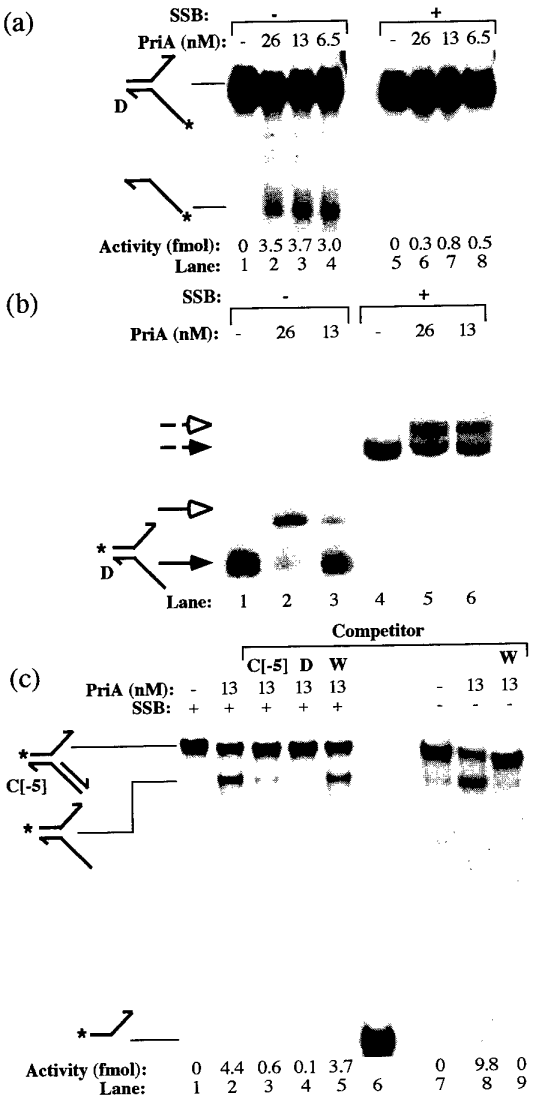


Figure 4. The effect of SSB on PriA fork binding and helicase activity. (a) Helicase assays using PriA at the concentrations indicated and SSB as indicated were conducted on substrate D (16 fmol) as described in Materials and Methods. PriA was added prior to SSB. The positions of starting substrate and labeled product are indicated. (b) Band-shift assays using PriA at the concentrations indicated and SSB as indicated were conducted on substrate D (16 fmol) as described in Materials and Methods. Filled/undashed arrow, free substrate; open/undashed arrow, substrate bound by PriA; filled/dashed arrow, substrate bound by SSB; open/dashed arrow, substrate bound by SSB and PriA. (c) Helicase assays on substrate C[−5] (16 fmol) were performed in the presence or absence of SSB (480 fmol) and 20-fold excess unlabelled competitor (C[−5], D or W). Substrate and competitor were incubated together with SSB prior to the addition of PriA.

ited PriA helicase activity on substrate D (Figure 4(a)), but PriA was able to form a supershift of substrate D in the presence of SSB (Figure 4(b), lanes 5-6). A competition experiment was performed to confirm that PriA could bind to substrate D in the presence of SSB. PriA unwinds the lagging-strand arm of substrate C[−5] at high activity levels in the presence of SSB (Figure 4(c), lane 2). The addition of 20-fold excess unlabeled substrate C[−5] or substrate D reduced the unwinding of labeled substrate C[−5] by at least 85% (Figure 4(c), lanes 3 and 4), while unlabeled substrate W had very little effect (Figure 4(c), lane 5). Substrate W was an effective competitor when SSB was not included in the reaction (Figure 4(c), lane 9). In reactions that included SSB, PriA was added to a mixture of substrate and competitor that had been pre-incubated with SSB. These data indicate that PriA can bind to substrate D even after it has been bound by SSB, but PriA is unable to initiate unwinding on substrate D in the presence of SSB regardless of whether PriA or SSB binds first. In contrast, the tailed duplex substrate W is only bound by PriA when SSB is absent.

On fork substrates, SSB inhibited unwinding of the duplex ahead of the fork but not the lagging-strand duplex, even when sufficient single-stranded DNA was available on the lagging-strand arm to allow SSB to bind. SSB influenced the polarity of helicase action on substrate C[−5]. When SSB was omitted (Table 1, line 5), the leading (S1-S2) and lagging (S2-S3) strand duplexes were unwound with nearly equal frequency. The presence of SSB inhibited the unwinding of the S1-S2 duplex in favor of unwinding of the S2-S3 duplex (Table 2, line 5). The S2-S3 duplex alone was unwound on 8 fmol out of the 9 fmol substrates that were consumed in this reaction. The effect of SSB on unwinding the S2-S3 duplex on substrate C with gaps of increasing size on the lagging-strand was complex. SSB enabled a small amount of unwinding of the S2-S3 duplex on substrate C[−1], possibly because SSB on the leading-strand arm caused some small, transient duplex opening at the fork junction (Figure 3(b), filled circles). SSB strongly stimulated unwinding of the S2-S3 duplex on substrate C[−5]. As the gap at the fork on substrate C was increased from five to 40 bases, the stimulatory effect of SSB on S2-S3 unwinding was diminished, but SSB did not abolish unwinding as it did on the duplex ahead of the fork (Figure 3(b), filled circles, and 3(c)). Even when SSB was added to substrate C[−40] prior to PriA, unwinding of the S2-S3 duplex continued to occur at high activity levels (Figure 3(c)). A gap of 40 bases is in theory large enough to be stably bound by SSB,[26] and SSB was able to inhibit the unwinding of substrate W, which had a single-stranded tail of 35 bases, and substrate D, which had a single-stranded leading-strand arm of 40 bases (Table 2).

The effect of SSB on PriA's ability to unwind substrates with gaps larger than 40 bases was

investigated using a new set of substrates. These were constructed by annealing various oligonucleotides to single-stranded M13Rt3 DNA. We first investigated activity on a "circular" substrate (substrate G) that was structurally equivalent to substrate C[−5] with a single-stranded leading-strand arm and a duplex lagging-strand arm. Substrate G was composed of the oligonucleotides S1 and S3[−5] annealed to M13Rt3. The annealing of S1 creates the forked structure needed to bind PriA to M13Rt3 DNA, and displacement of labeled S3[−5] was measured in helicase assays. PriA helicase was able to recognize and unwind substrate G very efficiently (Figure 5(a)), displacing the S3[−5] oligonucleotide on up to 2.6 out of 3 fmol total substrate. The helicase had relatively little activity on circular substrates H and I (Figure 5(b) and (c)), which lacked forked structures. Unlike the oligonucleotide substrates, helicase activity on all circular substrates tested was dependent on SSB (data not shown). This may be due to the presence of large excesses of single-stranded DNA which can be bound non-specifically by PriA.[30]

PriA efficiently unwound duplexes separated by gap of 80-2000 bases from the fork. To create substrates with larger gaps at the fork, oligonucleotide S3[−5] on substrate G was replaced with oligonucleotides that annealed 80, 1000 or 2000 bases from the S1 fork to create substrates J, K and L, respectively. PriA was able to dissociate the labeled oligonucleotide from all of these substrates even though SSB was added to the substrate prior to PriA (Figure 6(a)). These data indicate that SSB does not inhibit PriA translocation on the lagging-strand arm of the fork.

PriA helicase efficiently unwound duplexes of 300 bp but not 500 bp

Extension of the 3′ end of oligonucleotide S3[−5] on substrate G allowed us to examine helicase activity on duplexes of increasing length. PriA readily unwound duplexes of 150 and 300 bp on substrates M and N, respectively (Figure 6(b), lanes 1-4), consuming most of the substrate in 15 minutes in the presence of 2 mM ATP. Although PriA could easily translocate through a region of 2000 bases of single-stranded DNA (Figure 6(a)), unwinding activity was considerably reduced for duplexes of 500 and 1000 bp on substrates O and P (Figure 6(b), lanes 5-8). The unwinding of longer duplexes could not be further stimulated by increasing concentrations of ATP or PriA (data not shown). The ATP concentration required for half maximal unwinding ($[ATP]_{1/2}$) was 0.05 mM for duplexes as long as 150 bp, but increased to 0.1 and 0.2 mM for duplexes of 300 and 500 bp, respectively (data not shown). While these values are lower than those reported for duplexes of similar length,[31] the overall trend of increasing $[ATP]_{1/2}$ with increasing duplex length was maintained. This suggests that PriA is best suited to unwind duplexes of less than 500 bp.

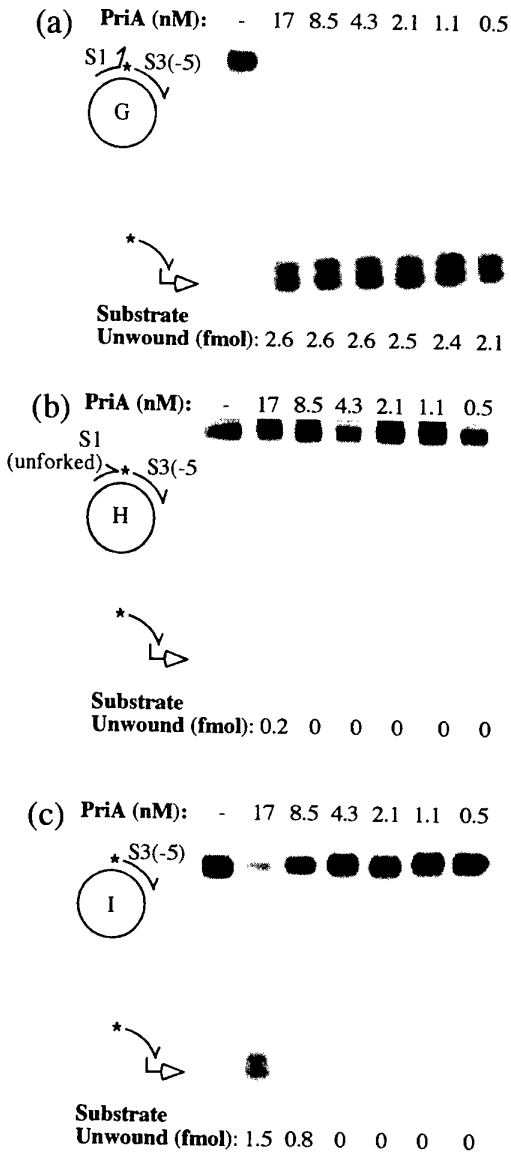


Figure 5. PriA unwinds a forked circular substrate, substrate G. Helicase assays using PriA at the concentrations indicated and SSB were conducted on 3 fmol substrate G (a), H (b) or I (c) as described in Materials and Methods. The region of duplex to be unwound was 65 bp long. The positions of starting substrate and dissociated oligonucleotide are indicated. The S3[−5] oligonucleotide runs as a doublet under these conditions. Substrate composition was as follows: substrate G (M13Rt3, S1, S3(−5)); substrate H (M13Rt3, S1(unforked), S3(−5)); substrate I (M13Rt3, S3(−5)). The labeled oligonucleotide is indicated with an asterisk (*).

Discussion

The interplay between PriA fork binding and helicase activities

PriA's vital role in initiating DNA replication on recombination intermediates and arrested replica-

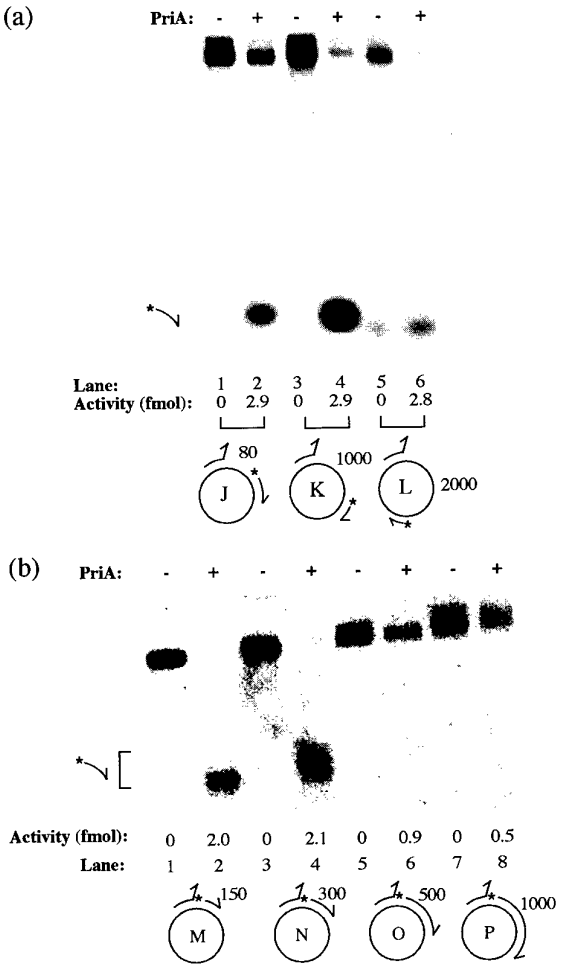


Figure 6. The effect of gap size and duplex length on PriA helicase activity on forked circular substrates. (a) Helicase assays using PriA (4.3 nM) and SSB were conducted on substrates J-L (3 fmol) as described in Materials and Methods with SSB being added to the reaction prior to PriA. The size of the gap(s) between the fork and additional oligonucleotides is indicated. The labeled oligonucleotide is indicated with an asterisk (*). Substrate composition was as follows: substrate J (M13Rt3, S1, −80); substrate K (M13Rt3, S1, −1000), substrate L (M13Rt3, S1, −2000). (b) Helicase assays using PriA (4.3 nM) and SSB were conducted on substrates M-P (3 fmol) as described in Materials and Methods. The lengths of the duplexes to be unwound were as follows: substrate M, 150 bp; substrate N, 300 bp; substrate O, 500 bp; substrate P, 1000 bp.

tion forks requires that it act on a variety of potential DNA substrates. While PriA's helicase and preprimosome assembly activities can be coupled,[22] only certain substrates such as the Mu fork and A-forks require duplex opening for preprimosome assembly and initiation of replication. Activation of the helicase is strongly influenced by fork structure and SSB. When a small region of single-stranded DNA is present, PriA helicase can translocate 3′ to 5′ on the leading or lagging-strand template to unwind the duplex ahead of the fork or the lagging-strand duplex, respectively (Figure 7(a) and (b)). We have previously found that on an A-fork (i.e. substrate C[−5]), PriA assembles the preprimosome on the same strand on which it translocates,[22] suggesting that only translocation on the lagging-strand template would lead to productive preprimosome assembly. When the single-stranded leading-strand arm of the A-fork is bound by SSB, translocation on this strand is blocked (Figure 7(c)). This confines helicase activity, and ultimately preprimosome assembly, to the lagging-strand arm (Figure 7(d)).

Nurse *et al.*[28] have proposed that PriA can bind to DNA in two modes. The first involves binding by PriA's helicase domain to the minimal substrate, a duplex with a 3′ single-stranded tail (similar to substrate W). The second, higher-affinity mode involves engagement of a specific fork-binding activity which is hypothesized to recognize bent DNA at the fork.[28] As an extension of this model we propose two modes of PriA helicase activity: basal (PriA[BAS]) and synergistic (PriA[SYN]). In the PriA[BAS] configuration, only PriA's helicase domain interacts with the substrate (Figure 7(b)). Basal PriA helicase activity results in the relatively poor unwinding of duplexes with 3′ tails such as substrate W, and it also is likely to account for the unwinding of the duplex ahead of the fork on substrates with single-stranded leading-strand arms (Figure 7(b)). In the more effective PriA[SYN] mode, synergism between PriA's helicase and fork-binding activities stimulates the unwinding of certain forked substrates. PriA[SYN] activity is most evident on the A-fork substrate (substrate C[−5]), where the unwinding of the lagging-strand duplex (S2-S3) is strongly stimulated relative to substrate W. It is likely that the relative orientation of the helicase and fork-binding domains are such that fork-specific binding brings the helicase domain into close proximity with the lagging-strand template of the fork (Figure 7(a) and (d)).

The PriA[BAS] and PriA[SYN] configurations can be distinguished by their sensitivities to SSB, which blocks only PriA[BAS]. While the presence of excess substrate W inhibited PriA helicase action on a radiolabeled substrate in the absence of SSB, only forked substrates inhibited helicase action in the presence of SSB. This indicates that SSB can inhibit fork-independent binding of PriA to DNA (Figure 7(c)). It is likely that *in vivo* the basal mode is largely suppressed by SSB. On the other hand, the observation that SSB does not inhibit PriA translocation on the lagging-strand indicates that PriA[SYN] can access single-stranded DNA despite the presence of SSB and/or that PriA in this configuration can displace the SSB (Figure 7(d)). PriA has been shown to displace SSB when helicase activity was initiated from a ϕX PAS.[32] Rapid translocation away from the fork, as we observed on substrates with large gaps on the lagging

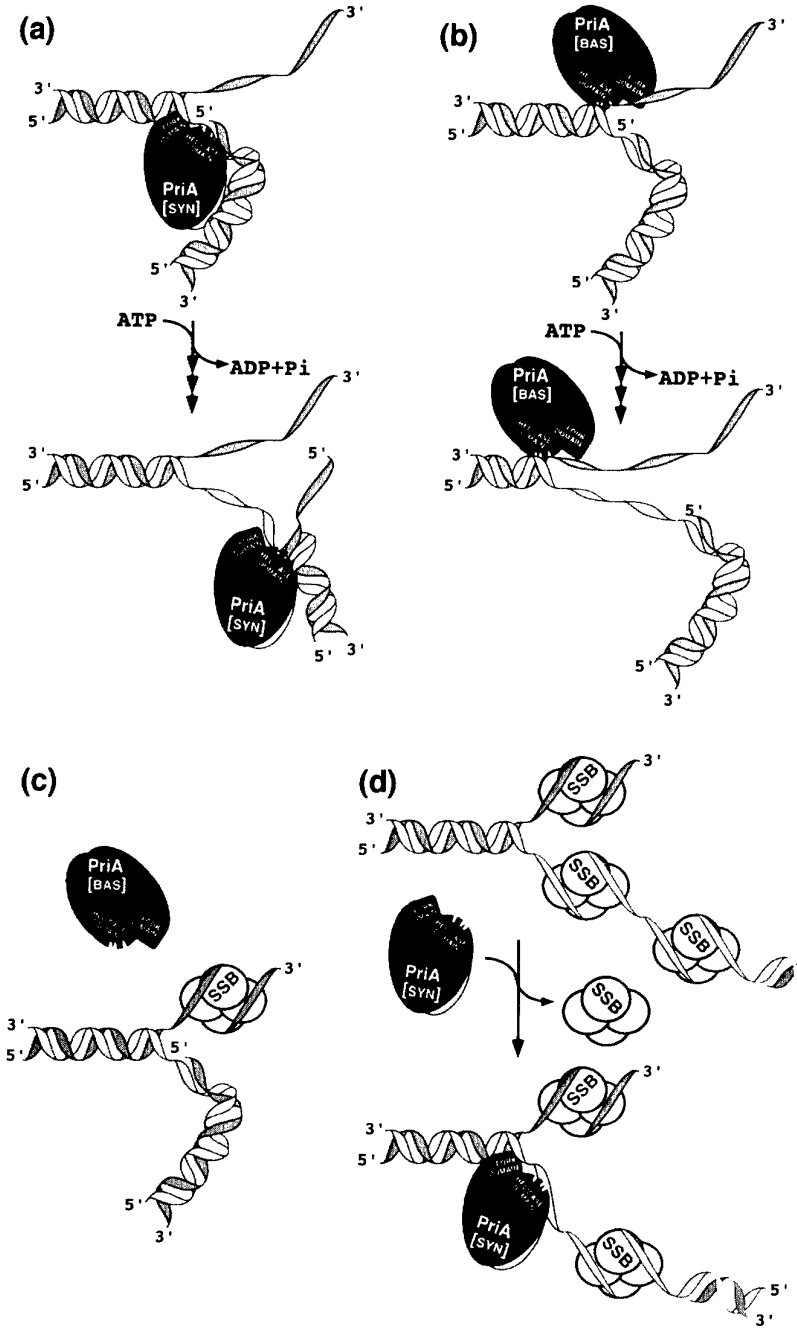


Figure 7. Synergism in unwinding the lagging-strand arm of an A-fork. (a) PriA is recruited to the A-fork based on the high affinity of the FORK DOMAIN for bent DNA. Binding to the lagging-strand (PriA[SYN]) places the HELICASE DOMAIN in the proper configuration to initiate unwinding of the lagging-strand arm. (b) The substrate is only bound by the HELICASE DOMAIN and not by the FORK DOMAIN in the basal mode (PriA[BAS]). This mode of unwinding is analogous to helicase action on duplexes with 3′-single-strand extensions. (c) SSB on the leading-strand arm prevents the binding of PriA to that arm by the basal mode (PriA[BAS]). (d) A large single-stranded gap on the lagging-strand arm coated with SSB does not prevent binding of the FORK DOMAIN to the fork. Binding to the fork promotes engagement of the HELICASE DOMAIN despite the presence of SSB. Binding and/or translocation could result in the transient displacement of SSB.

strand, might decrease the efficiency of primosome assembly and initiation of replication at the fork. Other primosome components have been shown to inhibit the initiation of PriA helicase activity on ϕX PAS substrates,[5] and we have observed a similar effect on substrates such as substrate J, the M13 DNA substrate with an 80-base gap between the fork and the oligonucleotide to be displaced (unpublished observations).

Additional factors may also be required to assist in PriA engagement during Mu replication. PriA helicase activity is required for high levels of phage Mu DNA replication,[22] but on the Mu fork it is the leading-strand template that is exposed by the small gap necessary to initiate unwinding. This structure would not help PriA unwind the lagging-strand arm of the fork, and it also would not provide a region of single-stranded DNA sufficient to allow for binding by SSB and suppression of unwinding by PriA[BAS]. Host factors ($MRF\alpha_2$) needed to make the transition from strand transfer by the phage transposase to replication by host fac-

tors could potentially play a key role in the suppression of the basal mode of unwinding. MRFα_2 could also promote initiation of unwinding of the lagging-strand duplex by PriA[SYN] by helping the helicase domain to gain access to the lagging-strand template.

Physiological significance of the PriA helicase

The comparison of PriA amino acid sequences from many bacterial species indicates that the helicase motifs have been highly conserved.[14] Despite this, genetic analyses have not yet indicated a critical role of PriA helicase activity in cellular metabolism. Mutant alleles lacking helicase activity (PriA K230R and PriA K230A) can restore apparently wild-type viability levels to *priA2::kan* strains and allow these strains to assimilate genetic markers by homologous recombination.[8,33,34] Work in the reconstituted phage Mu replication system indicates that other cellular proteins can compensate for the lack of PriA helicase activity.[22] However, when the helicase is intact these proteins are not required for Mu replication, suggesting that under normal circumstances PriA helicase and primosome assembly activities work together.

A growing body of evidence suggests that PriA's primary cellular role is to assist in the resumption of replication following replication-fork arrest. Such resumption is believed to occur by a variety of pathways.[12–14] PriA's helicase and primosome assembly activities may contribute to a rapid pathway for direct reactivation of the arrested replication fork.[14] In this pathway PriA would bind to the A-fork and unwind the lagging-strand arm, creating a binding site for DnaB while promoting assembly of the preprimosome.[22] Once loaded on the lagging-strand template, DnaB would coordinate assembly and activity of the processive replisome.[35]

If necessary, other proteins in the cell could compensate for the lack of PriA helicase activity by creating the duplex opening necessary for loading of DnaB helicase. Courcelle & Hanawalt[36] have found that RecQ and RecJ can process blocked replication forks, degrading nascent DNA on the lagging-strand arm, and inactivation of the Rep 3′ to 5′ helicase in a PriA K230R background results in a partial growth defect,[33] indicating that it may be partially redundant with PriA helicase. Alternatively, in the absence of PriA helicase activity, the resumption of replication may occur solely by pathways that depend on D-loop formation[12,13,37] as PriA-dependent assembly of a replisome on synthetic D-loops is supported by the helicase-inactive mutant PriA K230R.[24] However, this would leave the cell with fewer tools for coping with DNA damage and replication-fork arrest. Only the combined fork binding, primosome assembly, and helicase activities of PriA provide it with the flexibility to respond most appropriately to the varied needs of the cell.

Materials and Methods

Proteins

E. coli DNA polymerase I Klenow fragment [3′-5′ exo$^-$] and T4 polynucleotide kinase (PNK) were purchased from New England BioLabs. Purification of PriA and SSB has been described previously.[22] PriA concentration was determined by the method of Pace *et al.*;[38] SSB concentration was determined by the method of Bradford.[39] Molar concentrations were calculated with PriA as monomer and SSB as tetramer.

DNA substrates for band-shift and helicase assays

DNA substrates were constructed from the oligonucleotides shown in Figure 2 and below: −80, GGCATTTTCGGTCATAGCCCCCTTATTAGC; −1000, ATGACAACAACCATCGCCCACGCATAACCG −2000, AAAACGAGAATGACCATAAATCAAAAATCA; S1(unforked), CCATTAGCAAGGCCGGAAACGTCACCAATG. The composition of each substrate is provided in appropriate Table and Figure legends. Forked-oligonucleotide substrates (C, D, E, F and W) were constructed and purified as described.[22] Circular substrates (substrates G-P) were constructed by annealing various oligonucleotides to single-stranded M13Rt3, which does not contain a primosome assembly site.[22] To create the longer regions of duplex on substrates M-P, complexes (1 pmol) including end-labeled S3[−5] were purified by spin chromatography and incubated with *E. coli* Klenow fragment [3′-5′ exo$^-$] (1 U) for two to 20 minutes at 37 °C in buffer provided by the manufacturer and 0.1 mM of each dNTP. This resulted in extension of the S3[−5] 3′ end from about 100-900 bp as determined on a denaturing agarose gel, for a total duplex length of 150-1000 bp. Complexes were separated on a 1% (w/v) agarose gel in 1× TAE buffer[40] and purified by electroelution.

Helicase assays

Forked oligonucleotide substrates (0.8 nM) were combined in 20 mM Tris-HCl (pH 7.5), 5.4 mM $MgCl_2$, 1 mM DTT, 0.1 mg/ml BSA, and 2 mM ATP with PriA (13 nM) and SSB (12 nM) where indicated in a total volume of 20 μl. Reaction mixtures excluding SSB were incubated on ice for ten minutes. SSB was then added and the reactions were incubated for 15 minutes at 30 °C. In some cases the order of addition of these components was reversed such that SSB was pre-incubated with the substrate. Deproteinized products were separated on 10% (w/v) polyacrylamide gels (cross-linked at a ratio of 30:1) in TBE buffer[40] at 140 V for 2.5 hours. Gels were dried and subjected to phosphorimagery and autoradiography. For circular substrates (G-P) reactions were conducted in a similar manner with the exception that substrate was present at 0.1 nM and SSB was increased to 1 μM in a total reaction volume of 30 μl. Products were deproteinized with proteinase K and SDS and separated on 1% agarose gels in TAE buffer at 80 V for 2.5 hours. All experiments included a negative control (a reaction from which PriA was omitted) and markers representing potential helicase products. The amounts of total substrate converted to each product and total substrate consumed were calculated. Values presented in the text have been corrected for background (i.e. dissociation of substrate in the absence of PriA).

Band-shift assays

Band shifts were conducted essentially as described[22,27] using DNA substrates (0.8 nM), PriA (as indicated) and SSB (12 nM) as indicated in 20 μl reaction mixtures. As with the helicase assay, substrates were incubated with PriA for ten minutes on ice prior to the addition of SSB. Band-shift gels were dried and subjected to phosphorimagery and autoradiography.

Other

All quantification was by phosphorimagery using the Molecular Dynamics Storm 840 system and ImageQuant™ 1.11 B15 software.

Acknowledgments

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